Zoospore Density-Dependent Behaviors of *Phytophthora nicotianae* Are Autoregulated by Extracellular Products

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ABSTRACT

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Phytophthora species are destructive fungus-like plant pathogens that use asexual single-celled flagellate zoospores for dispersal and plant infection. Many of the zoospore behaviors are density-dependent although the underlying mechanisms are poorly understood. Here, we use *P. nicotianae* as a model and demonstrate autoregulation of some zoospore behaviors using signal molecules that zoospores release into the environment. Specifically, zoospore aggregation, plant targeting, and infection required or were enhanced by threshold concentrations of these signal molecules. Below the threshold concentration, zoospores did not aggregate and move

toward a cauline leaf of *Arabidopsis thaliana* (Col-0) and failed to individually attack annual vinca (*Catharanthus roseus* cv. Little Bright Eye). These processes were reversed when supplemented with zoospore-free fluid (ZFF) prepared from a zoospore suspension above threshold densities but not with calcium chloride at a concentration equivalent to extracellular Ca^{2+} in ZFF. These results suggest that Ca^{2+} is not a primary signal molecule regulating these communal behaviors. Zoospores coordinated their communal behaviors by releasing, detecting, and responding to signal molecules. This chemical communication mechanism raises the possibility that *Phytophthora* plant infection may not depend solely on zoospore number in the real world. Single zoospore infection may take place if it is signaled by a common molecule available in the environment which contributes to the destructiveness of these plant pathogens.

The genus *Phytophthora* comprises about 100 species of destructive pathogens that attack a broad range of economically important agricultural and ornamental crops as well as forest tree species (10,11,13). *Phytophthora* species produce flagellate single-celled zoospores as their primary dispersal and infection agents (6,9,23), and many zoosporic behaviors are density-dependent. For example, single zoospores seldom colonize even under optimal conditions (4). Zoospores aggregate only at or above 2 to $5 \times 10^5 \text{ ml}^{-1}$ for *P. palmivora* (31), 1 to $2 \times 10^5 \text{ ml}^{-1}$ for *P. capsici* (25), and $\geq 5 \times 10^5 \text{ ml}^{-1}$ for *P. infestans* (28). Similarly, plant infection by *Phytophthora* species requires certain densities of inoculum although the threshold is much lower compared with that needed for aggregation; this threshold density varies from species to species (12,24,28,29,37). It is not yet clear how such communal behaviors are regulated.

Calcium has been thought of as a regulating molecule responsible for many zoosporic behaviors since zoospores can produce this molecule during encystment (21,38), and many zoospore behaviors including autoaggregation and plant infection can be affected by exogenous calcium or calcium inhibitors (6,31,36,37). For example, cyst germination can be induced by CaCl_2 or a zoospore-free suspension which may contain calcium released from zoospores (5). This process may be inhibited by calcium antagonists, then restored by adding CaCl_2 (38). Similarly, zoospore aggregation is thought to be calcium-dependent because addition of calcium antagonists to a zoospore suspension inhibits this process (31), although it is not known whether this process is affected directly by calcium. Effects of calcium on some zoospore

behaviors are concentration-dependent; higher concentrations suppress zoospore motility (3) and hamper plant diseases caused by *Phytophthora* (37), while lower concentrations induce zoospore production, cyst germination, and adhesion (8,15,16).

In this study, we use *P. nicotianae* (synonym: *P. parasitica*) as a model to investigate regulation of zoospore communal behaviors. *P. nicotianae* infects more than 800 plant species and causes root and foliage damage although it has not been reported to infect *Arabidopsis* plants (10,33). Zoospore plant infection is known to be associated with concentration of inocula, and zoospore aggregation is thought to be a factor that can increase the infection potential (19,37). We hypothesize that zoospore aggregation, plant targeting, and infection behaviors are coordinated through zoospore extracellular products. We demonstrate that zoospores detect and respond to the extracellular products in zoospore-free fluid (ZFF) to regulate their communal behaviors. The effect of calcium as a signal molecule for zoospore aggregation and plant infection is also examined. This study advances our understanding of chemical communication as a potentially important factor in the infection of plants by *Phytophthora* species.

MATERIALS AND METHODS

Preparation of ZFF. *P. nicotianae* isolate 1B11 was used for ZFF preparation. First, the 1- to 2-week-old culture on clarified 20% V8 agar (CV8) (32) was cut into 5-mm² plugs, and two plugs were added to each 90-mm-diameter petri dish with 10% CV8 broth and incubated at 23°C in the dark for 2 weeks to induce production of sporangia. After the seed plugs and medium were removed, the mycelial mats were rinsed five times with sterile distilled water (SDW) to eliminate nutrients from the remaining medium. The drained mycelial mats were incubated for 1 week under fluorescent light at 23°C. For zoospore release, the mats with numerous sporangia were rinsed an additional five times with SDW and then flooded with 8 ml of chilled SDW and kept

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*The e-Xtra logo stands for "electronic extra" and indicates that Figure 3 appears in color online.

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under light for 1 h. Zoospore density was usually 10^6 ml^{-1} unless otherwise stated. All procedures were performed under sterile conditions to prevent bacterial contamination. Finally, to obtain ZFF, zoospore suspensions were filtered through sterile miracloth to remove mycelia, sporangia, and other structures, followed by vortexing briefly to facilitate chemical release (21). The suspensions were then filtered through a $0.2\text{-}\mu\text{m}$ syringe filter to remove the cysts. ZFF was used fresh or from storage at -20°C .

Fresh zoospore production for microscopy and inoculation assays. To produce zoospores rapidly, a dozen culture plugs were flooded with 1% sterile soil-water extract in a 90-mm-diameter petri dish for 8 to 16 h. The plugs were then rinsed three times with SDW and placed under fluorescent lights overnight to induce sporangium production. To facilitate zoospore release from the sporangia and eliminate nutrients from the plugs, the plugs were further rinsed three times with chilled SDW and incubated in SDW for up to 1 h. Zoospore density was determined using a hemacytometer under a light microscope, and the suspension was diluted with SDW to a stock concentration of 10^4 zoospores ml^{-1} for further applications.

Analysis of zoospore aggregation. Zoospore aggregation was examined under an IX71 inverted microscope (Olympus America Inc., PA). In preliminary studies, zoospores in SDW did not aggregate at densities below $5 \times 10^5 \text{ ml}^{-1}$. To determine the effects of ZFF and calcium on zoospore aggregation, zoospore suspensions at $2 \times 10^3 \text{ ml}^{-1}$ were used. Suspensions were made by mixing 30 μl of zoospore suspension stock at 10^4 zoospores ml^{-1} with 120 μl of a test solution in a 150- μl depression slide well. Test solutions included ZFF, dilution series of CaCl_2 from 10 mM, and $10^4 \times$ diluted CV8 broth. Slides with zoospore suspensions were incubated on wet filter paper in 10-cm-diameter petri dishes at 23°C . Zoospores in two wells were examined under the microscope for activity in six fields at 1, 4, 16, 24, and 48 h of incubation. Zoospores that aggregated or formed clumps in all three observation fields per well were recorded as aggregation positive. Zoospore motility and germination rates were also recorded. Images were captured with the Image-Pro Plus software version 5.1 (Media Cybernetics, Inc., MA). Each test was done twice.

Analysis of zoospore plant targeting. The effect of ZFF on zoospore chemotaxis to plants was tested by exposing a low density of $1.6 \times 10^3 \text{ ml}^{-1}$ of zoospores to plant tissue. An aliquot of 40 μl of fresh zoospore-suspension stock at 10^4 ml^{-1} was mixed with 210 μl of ZFF or SDW in a well of 24-well plates. A detached cauline leaf from 6-week-old *Arabidopsis thaliana* (Col-0) seedlings grown in Metro Mix 200 (Sun Grow Horticulture, Bellevue, WA) in a growth chamber was then added to the suspension. The treatment plates were kept at 23°C and zoospore development was examined over a period of 5 days. Images were captured as described for aggregation tests. The test included three wells per treatment and was done three times.

Zoospore plant infection assay. Several assays were performed with annual vinca (*Catharanthus roseus* cv. Little Bright Eye). Four-week-old seedlings were transplanted in pine bark and then grown for 4 to 6 weeks in a greenhouse. Prior to inoculation, leaves were detached from plants, surface-sterilized with 70% ethanol, and rinsed in SDW to minimize potential interference from other microbes before placement on glass rods in plastic containers. All inoculations were done by applying ten 10- μl drops of inoculum to different sites on the underside of each leaf. To determine zoospore density dependence of plant infection, a fresh zoospore-suspension stock at 10^4 ml^{-1} was applied undiluted or $10 \times$ or $100 \times$ diluted with SDW (i.e., 10 and 1 zoospore per 10- μl drop). To examine the effects of extracellular products, Ca^{2+} , and nutrient residues from media, the zoospore suspension stock was diluted with a test solution to 100 ml^{-1} so that each 10- μl drop of inoculum would contain approximately one zoospore. Test solutions included ZFF, dilution series of CaCl_2 from 10 mM, $10^4 \times$ diluted CV8 broth, and SDW. Inoculated

leaves were kept in a moist chamber and incubated in the dark overnight at 23°C followed by an additional 3 days with a 10-h/14-h day/night cycle in a growth chamber. Inoculated sites developing water-soaked lesions during the 72 h incubation time were recorded as infected. Each test included six replicate leaves and was done three to six times. The disease incidence data from these tests were pooled after analysis of homogeneity and then analyzed to determine the significance of difference among treatments using analysis of variance (SAS Institute, Cary, NC).

RESULTS

Extracellular products mediate zoospore aggregation. As illustrated in Figure 1A, zoospores in SDW aggregated only at $5 \times 10^5 \text{ ml}^{-1}$ or higher densities. To examine whether the auto-aggregation is mediated through molecules released from zoospores, a fresh zoospore suspension stock was diluted to $2 \times 10^3 \text{ ml}^{-1}$ with ZFF, CV8 broth, or SDW. At this density, 250-fold lower than the threshold, only those zoospores in ZFF aggregated. Zoospores gradually moved together and formed clusters of cysts (Fig. 1B and C). In contrast, zoospores in SDW (Fig. 1D and E) or diluted CV8 broth at the same density did not aggregate. Many of the zoospores remained swimming after 4 h incubation in SDW (Fig. 1D). More than 30% of zoospores in the suspension germinated in the diluted CV8 after overnight incubation (Fig. 1F). These results indicate that zoospore aggregation was coordinated chemically by zoospore-extracellular products rather than nutrient residues from media.

Extracellular products affect zoospore plant targeting. Zoospores target plants by chemotaxis. Previously this was attributed to chemotactic substances from plants (6). To determine whether zoospores themselves play a role in this process, activities of *P. nicotianae* zoospores in SDW or ZFF at a low density of $1.6 \times 10^3 \text{ ml}^{-1}$ were examined in the presence of an *Arabidopsis* cauline leaf. Clumps of encysted zoospores or cysts were observed on the leaf surface, especially on trichomes, in wells containing ZFF after 4 h incubation (Fig. 2A). In contrast, zoospores at the same density and incubation time did not cluster on plant tissue in control wells containing only SDW; they scattered randomly in the wells (Fig. 2B). This indicates that zoospore taxis to and settlement on plant tissue was mediated through their own extracellular products instead of those from plants. Comparison of cyst germination with and without plant tissue implies that plant exudates induced germination of cysts irrespective of presence or absence of ZFF. With plant tissue the germination rates were up to 90%, while without plant tissue few cysts germinated in either ZFF (Fig. 1B and C) or SDW (Fig. 1D and E) even with further incubation. More germlings were observed on the leaf surface in presence of ZFF which produced sporangia in or on the plant tissue after 5 days of incubation (Fig. 2C). This was dramatically different from incubation of the same numbers of zoospores in SDW (Fig. 2D). These results indicate that extracellular products of zoospores played a primary role in zoospore taxis to and infection of plant tissue.

Extracellular products regulate plant infection by zoospores. Plant infection by *P. nicotianae* in SDW was density-dependent (Fig. 3A). The infection rate on the inoculation sites dropped with decreasing number of zoospores per site, and single-zoospore inoculation resulted in little infection. More than 20% of the sites did not develop any symptoms with 100 zoospores per site, indicating the requirement of a higher density of zoospores for an optimal infection rate under this condition.

To determine whether zoospore infection is mediated by a chemical communication process through signaling molecules from zoospores, single zoospore inoculation was conducted. After 72 h incubation, few sites inoculated with single zoospores in diluted CV8 or SDW developed a lesion (Fig. 3B [left] and C, Fig. 4D). In contrast, more than 70% of sites inoculated with

single zoospores in ZFF developed lesions. This rate was nearly as high as that obtained with 100 zoospores in SDW (Fig. 3B). When ZFF was diluted, plant infection rates from single-zoospore inoculation decreased (Fig. 3C). Thus, a single zoospore may infect a plant as long as there is adequate signals in the environment.

Effects of exogenous Ca^{2+} on zoospore communal behaviors. To determine whether Ca^{2+} is used for communication of zoospores in zoospore aggregation and plant infection, CaCl_2 at different concentrations was tested as was done for ZFF. Unexpectedly, zoospores did not aggregate in CaCl_2 solutions at any of the concentration range tested (Fig. 4B and C). Most cysts, irrespective of Ca^{2+} concentration, scattered in the wells of depression slides and germinated after overnight incubation as in diluted CV8 broth (Fig. 1F). The germination rate decreased with decreasing concentrations of Ca^{2+} .

Similarly, inoculation with single zoospore in CaCl_2 solutions resulted in infection of fewer sites on detached annual vinca leaves compared with that in ZFF. The infection rate decreased with decreasing Ca^{2+} concentration (Fig. 4D). The highest infection rate, obtained with CaCl_2 at 10 mM, was significantly ($P < 0.0001$) lower than the rates with ZFF (20% versus 65%). The Ca^{2+} concentration in the ZFF should be no more than

500 nM as calculated according to a previous study (38). Therefore, molecules other than Ca^{2+} in ZFF were responsible for signaling zoospore aggregation and plant infection.

DISCUSSION

Density-dependent behavior in bacteria is controlled by a process called “quorum sensing,” a cell-to-cell communication mechanism by which bacteria count their own numbers by producing and detecting the accumulation of a signaling molecule or autoinducer that they release into the environment (14,39). Such a mechanism controls bacterial processes including biofilm formation, virulence-factor secretion, and sporulation as well as pathogenicity and plant colonization (34). Quorum sensing also has been documented in fungi and regulates processes such as dimorphism and biofilm formation, although fungal species use completely different autoinducers from those employed by bacteria (18). Processes controlled by quorum sensing have one thing in common: they are unproductive when undertaken by a single individual but become effective when undertaken by the group. They are all coordinated by the extracellular products or autoinducers (39). This study has demonstrated that quorum sensing operates in *P. nicotianae*, regulating zoospore aggrega-

Fig. 1. Effect of zoospore-free fluid (ZFF) on zoospore aggregation of *Phytophthora nicotianae*. Zoospore suspension was incubated in depression slide wells containing a test solution of 150 μl at 23°C. Zoospores in sterile distilled water (SDW) at $2 \times 10^6 \text{ ml}^{-1}$ aggregated after **A**, 16-h incubation. Zoospores in ZFF at $2 \times 10^3 \text{ ml}^{-1}$ aggregated after **B**, 4 h and **C**, 16 h incubation, but not in SDW at the same density and incubation times (**D** and **E**), nor did those in $10^4\times$ diluted CV8 broth medium after **F**, 16 h. Six fields were examined for each treatment point. Images were captured from representative fields under an inverted microscope. Bar = 50 μm . *c* = cyst, *mz* = motile zoospore, *ac* = aggregated cyst, *g* = germinant.

tion, targeting, and infection of annual vinca and *Arabidopsis*. These data support the contention that cell chemical communication is a fundamental microbiological mechanism operating beyond the bacterial world (1).

We provide the first experimental evidence that zoospore chemical communication is an important factor in the infection process. *P. nicotianae* zoospores in the ZFF can aggregate on the leaf surface at a low density (Fig. 2A) and subsequently germinate and penetrate plant tissue (Fig. 2C). They do not target or attack a plant when the signal molecules are below the threshold level. When chemical signals in the environment reach the threshold concentration, zoospores initiate the taxis and infection processes. These molecules from zoospores and maybe other structures of *Phytophthora* are functionally different from those in plant exudates or nutrients that stimulate cyst germination (7). They serve as autoinducers which regulate zoospore aggregation, taxis, and infection.

Chemical communication may give zoosporic plant pathogens advantages in their fitness and pathogenesis. For example, it may direct *P. nicotianae* zoospores to work synchronically as they target plants and aggregate on the plant surface (Fig. 2A) (17). Such team work will subsequently facilitate plant infection. How-

ever, how this communication mechanism works to the advantage of *Phytophthora* species in the real world remains unclear. *P. nicotianae* constantly causes disease problems in nurseries, but it was infrequently detected in its primary inoculum sources (e.g., in irrigation water) because of its low population density (2,20,26). The lack of zoospore detection before disease epidemics may imply the unlikelihood of aggregation as a mechanism for primary infection. Aggregation may only facilitate further cycles of infection as zoospores accumulate after sporangium production in infected plant tissue at a later stage of infection (37). Alternatively, aggregation may serve other roles in zoosporic pathogen biology, such as biofilm formation for drug resistance as described for bacterial and fungal species (30), which deserves further investigation.

The lack of zoospore detection before infection also raises a question about the ability of zoospores at low density to infect plants in the environment. There are three possibilities. First, the pathogens may increase the concentration of the autoinducer by increasing zoospore numbers in the environment. Zoospore number before infection can be increased through secondary zoospore production as that occurs in irrigation water systems (35). Zoospore number can also be increased through disease cycles.

Fig. 2. Effect of zoospore-free fluid (ZFF) on zoospore plant targeting. *Phytophthora nicotianae* zoospore suspension at $1.6 \times 10^3 \text{ ml}^{-1}$ was incubated at 23°C in wells of a 24-well plate, each containing a test solution of $250 \mu\text{l}$. Zoospores in ZFF aggregated and encysted on a cauline leaf of *Arabidopsis thaliana* (Col-0) after **A**, 4 h and **C**, differentiated after 5 days, in contrast with zoospore behaviors in sterile distilled water at the same time points (**B** and **D**). Six fields were examined for each treatment point. Images were captured from representative fields under an inverted microscope. Bar = $50 \mu\text{m}$. at = *Arabidopsis* leaf tissue, c = cyst, e = exudates from plant tissue, h = hypha or germling, tc = trichome, sp = sporangium.

Once primary infection succeeds, thousands of zoospores will release from diseased plant tissue (10). Second, cysts around or on plant tissue may not attack a plant immediately but they may weaken plants so that other structures from them, such as infection hyphae, may directly infect plants. We observed exudates releasing from tissue incubated in not only ZFF but also SDW with a low density of zoospores (Fig. 2C and D). The infection by zoospores in SDW was delayed or attenuated and did not result in sporangia 5 days after treatment. Third, the chemical signals for zoospore communication and plant infection may already exist in natural environments. We recently found that AI-2, a universal autoinducer of bacteria, can be produced by plant zoosporic pathogens (27). This suggests that AI-2 available in environments may be shared by these pathogens if this signaling molecule can be used to regulate communal behaviors of zoosporic oomycetes, which remains to be determined.

Fig. 3. Effect of zoospore density and concentration of zoospore-free fluid (ZFF) on zoospore plant infection. Ten 10- μ l drops of *Phytophthora nicotianae* zoospore inoculum prepared in ZFF were placed at 10 different sites on the underside of each leaf of annual vinca (*Catharanthus roseus*). Inoculated sites were assessed for infection after 3-day incubation at 23°C. **A**, Density-dependent infection by zoospores suspended in sterile distilled water (SDW). Each column is a mean percentage of sites diseased from 36 replicate leaves in six independent tests and is topped with a standard error bar. **B**, Blight symptoms on a representative leaf inoculated with 10 drops of ZFF containing approximately one zoospore each compared with those on the leaf inoculated with 10 drops of SDW containing 1 or 100 zoospores each. **C**, Decreased infection with dilution of ZFF. Each column is a mean percentage of sites diseased from 36 replicate leaves in six independent tests and is topped with a standard error bar.

In addition to AI-2, other molecules may also act as auto-inducers for zoospore chemical communication in oomycetes. Calcium, for example, is produced by zoosporic pathogens and is also widespread in nature. However, in this study, we found that the processes stimulated by Ca^{2+} were different in many ways from those triggered by ZFF. We examined the effect of Ca^{2+} on zoospore aggregation and plant infection by addition of CaCl_2 at different concentrations to a zoospore suspension. First, we found that CaCl_2 did not induce zoospore autoaggregation at any concentration tested (Fig. 4A to D). Second, CaCl_2 did not trigger single-zoospore infection at the concentration equivalent to extracellular Ca^{2+} in ZFF (Fig. 4D). In contrast, it stimulated cyst germination and induced a low level of plant infection by *P. nicotianae* at a Ca^{2+} level 20,000 $\times$ higher than that in ZFF, which was shown to contain 500 nM Ca^{2+} when the zoospore density was 10^6 ml^{-1} (38). These results suggest that released extracellular

Fig. 4. Effect of Ca^{2+} on zoospore behavior and plant infection by *Phytophthora nicotianae*. Zoospores were incubated in a 150- μ l test solution at $2 \times 10^3 \text{ ml}^{-1}$ in depression-slide wells at 23°C. Zoospores in **A**, sterile distilled water (SDW), **B**, CaCl_2 at 1 μM , and **C**, 10 mM overnight did not aggregate. Six fields were examined for each treatment point. Images were captured from representative fields under an inverted microscope. Bar = 50 μm . **c** and **g** indicate cyst and germings, respectively. **D**, Comparison of infection rates by single zoospores in zoospore-free fluid (from zoospore suspension at $5 \times 10^5 \text{ ml}^{-1}$), $10^4 \times$ diluted CV8 broth, CaCl_2 dilutions, and SDW. Ten 10- μ l drops containing a single zoospore each were placed at 10 different sites on the underside of each leaf of annual vinca (*Catharanthus roseus*). Each column is a mean percentage of sites diseased from 18 replicate leaves in three independent tests and is topped with a standard error bar.

Ca²⁺ is not responsible for the regulation of communal behaviors in terms of Ca²⁺ concentration. However, Ca²⁺ has been proposed as a secondary messenger in chemical communication of *Ulva intestinalis* zoospores using a bacterial AHL autoinducer (22), and aggregation can be abolished using Ca²⁺ antagonists (31), the precise role of Ca²⁺ in *Phytophthora* zoospore-communal behavior is yet to be determined.

The chemical components of ZFF beyond AI-2 and Ca²⁺ are under investigation. ZFF was carefully prepared to eliminate nutrients from media and bacterium contamination. Mycelial mats in culture media were rinsed thoroughly before and after sporangium induction. The dilution factor by SDW in the CV8 broth was 10⁹ at the time of zoospore release (27). The concentration of CV8 broth used in zoospore aggregation and single zoospore infection assays was 10⁴, 10⁵ × higher than what may be present in ZFF. The diluted CV8 stimulated zoospore germination similar to CaCl₂ but did not stimulate aggregation and single zoospore infection (Figs. 1F and 4D). In fact, the fresh zoospore suspensions prepared for all the assays also contained minimum medium residues, but these zoospores were not active in aggregation, plant targeting and infection until ZFF was added. To determine the effect of other factors such as miracloth and filters, we compared ZFF supernatant prepared by centrifugation at 10,000 rpm with ZFF prepared by filtration. No differences were observed between these two preparations (data not shown), demonstrating that the active components in ZFF originated from *P. nicotianae*, not from culture media or other exogenous factors. Identification of the specific autoinducer responsible for communal behaviors of *P. nicotianae* is warranted to uncover the exogenous signal source affecting zoospore chemical communication and the mechanism of plant infection by zoospore pathogens in nature, which could facilitate the development of new control strategies for these pathogens.

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